INFECTION AND IMMUNITY, Apr. 2007, p. 2063–2066 Vol. 75, No. 4
0019-9567/07/$08.00+0 doi:10.1128/IAI.01565-06
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

NOTES

The Putative Glycosyltransferase-Encoding Gene *cylJ* and the Group B Streptococcus (GBS)-Specific Gene *cylK* Modulate Hemolysin Production and Virulence of GBS▽†

Marie-Pierre Forquin,[1] Asmae Tazi,[2] Manuel Rosa-Fraile,[3] Claire Poyart,[2] Patrick Trieu-Cuot,[1] and Shaynoor Dramsi[1]*

Unité de Biologie des Bactéries Pathogènes à Gram-Positif, Institut Pasteur, URA CNRS 2172, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France[1]; INSERM 4567-umR CNRS 810, Institut Cochin, Université Paris Descartes, Paris, France[2]; and Microbiology Service, Virgen de las Nieves University Hospital, Granada, Spain[3]

Received 28 September 2006/Returned for modification 3 December 2006/Accepted 21 January 2007

Group B streptococcus (GBS) expresses a hemolysin/cytolysin that plays an important role in pathogenesis. Using the *Himar1* transposon mutagenesis system, a hypohemolytic mutant carrying an interrupted *cylJ* gene was characterized. *cylJ*, encoding a putative glycosyltransferase, and *cylK*, whose product is unknown, are both required for the full hemolytic/cytolytic activity, pigment formation, and virulence of GBS.

Streptococcus agalactiae (also known as group B streptococcus [GBS]) is a commensal organism found in the gastrointestinal and genitourinary tracts of healthy individuals. However, in certain circumstances, mostly in neonates, GBS can become a life-threatening pathogen, causing invasive infections such as pneumonia, sepsis, and meningitis. The hemolysin/cytolysin expressed by GBS is an important virulence factor (8). Using IS*S1* transposon mutagenesis, Spellerberg et al. identified a cluster of genes that are required for the hemolytic activity of *S. agalactiae* (16). This cluster comprises at least 12 open reading frames that appear to belong to a single operon called *cyl* (15). It comprises genes encoding an ABC transporter (*cylA*, *cylB*), an acyl carrier protein homologue (*acpC*), additional proteins involved in fatty acid biosynthesis (*cylD*, *cylG*, *cylZ*, and *cylI*), a putative aminomethyltransferase (*cylF*), and a putative glycosyltransferase (*cylJ*); it also comprises three GBS-specific genes (*cylX*, *cylE*, and *cylK*) of unknown function. The ABC transporter encoded by this operon (*cylA* and *cylB*) was proposed to be required for hemolysin export at the bacterial surface (16). *cylAB* was recently shown to encode an ABC-type multidrug-resistant (MDR) transporter (5). Until now, 8 out of 12 genes composing the *cyl* operon have been insertionally inactivated (*acpC*, *cylZ*, *cylA*, *cylB*, *cylE*, *cylF*, *cylI*, and *cylK*), and the corresponding mutants all displayed a hypohemolytic phenotype, except for the *cylE* mutant, which clearly was nonhemolytic (5, 13, 17). Indeed, the gene *cylE*, which confers a hemolytic activity to the recombinant *Escherichia coli* strain, was identified as the structural gene for the GBS hemolysin/cytolysin (13). Using the *Himar1* transposon mutagenesis system developed for *Streptococcus equi* (7), a bank of about 2,000 mutants was generated in GBS strain NEM316. Efficient and random transposition of the minitransposon onto the chromosome of NEM316 was demonstrated by Southern blot analysis and insertion site sequencing of 24 randomly chosen mutants (data not shown). Screening of this bank on Todd-Hewitt agar containing 5% defibrinated horse blood revealed one hypohemolytic mutant in which the transposon had inserted in the 3′ end of *cylJ*, the penultimate gene of the *cyl* operon (Fig. 1). Quantitative reverse transcription-PCR analysis revealed that transcription of the downstream gene *cylK* was abolished in the *cylJ*::*Himar1* mutant (data not shown). This result indicates that *cylJ* and *cylK* are cotranscribed. In this work, we have analyzed the contributions of *cylJ* and of the downstream *cylK* gene to the hemolytic/cytolytic activity, its associated phenotype (i.e., pigment production), and GBS virulence.

Variations of hemolysin production in clinical isolates are difficult to quantitate precisely due to low levels of hemolysin activity in culture supernatants. To circumvent this problem, we deleted the CovR binding site in the *cyl* promoter region (bp 661363 to 661423 in the NEM316 genome, as defined at http://genolist.pasteur.fr/SagaList/), since CovR acts as a repressor of the *cyl* operon (6, 7). As expected, a 50-fold increase in β-galactosidase activity was observed in NEM316 when the mutated promoter was cloned into pTCV-*lac*, in comparison with the β-galactosidase activity of the wild-type *cyl* promoter (data not shown). The resulting mutant strain, strain CCH206, thereafter called Pcyl+, displayed a hyperhemolytic and hyperpigmented phenotype similar to that of the CovSR$^-$ mutant (data not shown). Therefore, to evaluate the respective roles of

* Corresponding author. Mailing address: Unité de Biologie des Bactéries Pathogènes à Gram-Positif, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: 33 1 44 38 94 87. Fax: 33 1 45 68 89 38. E-mail: sdramsi@pasteur.fr.

† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 5 February 2007.

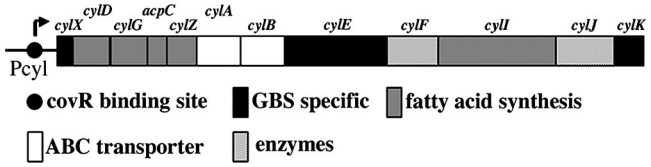

FIG. 1. Structure of the *cyl* operon in the serotype III GBS strain NEM316 (3). The 12 genes belonging to this cluster are shown, and similarities of the deduced proteins to gene products with known functions in the databases are indicated by shading. The CovR binding site in the promoter region is depicted.

cylJ and *cylK* in the hemolytic/cytolytic activity of *S. agalactiae*, in-frame deletions of these genes were constructed in a CCH206 genetic background (Δ*cylJ* [NEM2457] and Δ*cylK* [NEM2458], respectively). An in-frame deletion of *cylE* was also carried out in CCH206 (Δ*cylE* [NEM2456]) to further characterize this mutation in the same hyperhemolytic genetic background. The Δ*cylJ*, Δ*cylK*, and Δ*cylE* deletions were also constructed using the wild-type NEM316 strain, and similar results were obtained (data not shown).

The primers used for the construction of deletion alleles are listed in Table 1. In-frame deletions of *cylE* (using primers O1-O2 and O3-O4), *cylJ* (O5-O6 and O7-O8), and *cylK* (O9-O10 and O11-O12) were constructed by using splicing-by-overlap-extension PCR. A similar strategy was employed to delete the CovR binding site in the *cylX* promoter region (using primers O13-O14 and O15-O16). Chromosomal gene exchanges were carried out as described previously (1, 2). In-frame deletions of all genes were confirmed by PCR and sequence analysis. Macroscopic analyses of the mutants, colony morphology, and growth curves in complex Todd-Hewitt medium and in RPMI medium (used as a chemically defined minimal medium) did not reveal any differences from the parental strain. As shown in Fig. 2A, the Δ*cylE* strain is nonhemolytic, whereas the Δ*cylK* and Δ*cylJ* strains are significantly less hemolytic than the parental strain on a horse blood agar plate. GBS hemolysin was reported to act as a cytolysin on lung epithelial cells (9). We thus measured the cytotoxic activity of the different mutants on A549 human pulmonary epithelial cells as described previously (11). Release of the cytoplasmic lactate dehydrogenase (LDH) enzyme was used as an indicator of cell lysis. The Δ*cylE*, Δ*cylJ*, and Δ*cylK* mutants were found to be noncytotoxic (Fig. 2C), whereas the parental strain shows a time-dependent cytotoxic effect. Taken together, these results demonstrate that CylJ and CylK are required for full expression of the hemolytic/cytolytic activity of *S. agalactiae*, whereas only CylE appears essential to promote hemolysis.

Until now, in GBS, hemolysis and pigment production have never been dissociated (6, 9, 12, 16, 17). The GBS pigment has been recently identified as a glycopolyene (14). The detection of orange-red pigmented colonies in Granada medium is an easy way to screen and identify GBS in clinical laboratories (13). We therefore analyzed pigment production of the different mutants on Granada medium plates incubated under anaerobiosis at 37°C (Fig. 2B). The Δ*cylE* mutant was clearly not pigmented, whereas the Δ*cylK* and Δ*cylJ* mutants produced lesser amounts of pigment than did the parental strain. The degree of pigmentation correlates with the hemolytic activity of the mutants, with the Δ*cylE* mutant < the Δ*cylK* mutant < the Δ*cylJ* mutant < Pcyl+. Spectral analysis of pigment extract from GBS cultivated in Todd-Hewitt broth supplemented with 0.1% starch and 1% glucose at 37°C (6, 17) shows a characteristic triple peak in the parental strain that was reduced in the Δ*cylJ* mutant and absent from the Δ*cylE* and Δ*cylK* mutant extracts (Fig. 2D). Thus, the amplitude of spectral absorbance correlates with the level of hemolysin activity of the strains. To be certain of the essential role of the GBS-specific *cylK* in hemolytic activity and pigment biosynthesis, single-gene complementation was performed. The *cylK* gene was amplified in its entirety with primers O17-O18 (Table 1) and cloned into the low-copy-number shuttle vector pTCV-*erm* to be transcribed from the gram-positive kanamycin resistance gene promoter PaphA-3 (11). The vectors pTCV-*erm* and pTCV-*erm* (PaphA-3-cylK) were introduced by electrotransformation in NEM2458 (Δ*cylK*). Transcomplementation with a plasmid-borne *cylK* restored pigment production and hemolytic activity in the Δ*cylK* deletion mutant (see Fig. S1 in the supplemental material). These results point out the role of CylJ and CylK in

TABLE 1. Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)	Gene target
O1	GGGATCGAATTCTTAGGGACTGTTTTATCTGCGGCG	*cylE*
O2	GCGGCAGCACCCGGGCGTGCCAGTTAAAGGCCTACCGTCTGG	*cylE*
O3	GGCACGCCCGGGTGCTGCCGCATCCGCCAAGGGGAACATGTC	*cylE*
O4	ATAAGGATCCAAAGCTTTATGACTAGCCAACC	*cylE*
O5	TTCAGCAGAATTCCTCTCTCGTCAAGCATTGGA	*cylJ*
O6	GCGGCAGCACCCGGGCGTGCCTCCAAGGATATGAATATCATGTCC	*cylJ*
O7	GGCACGCCCGGGTGCTGCCGCGCGGAGGCAGAAATTGAATCCTTT	*cylJ*
O8	CTATAGGGATCCCACATTTATAGGAAATTTC	*cylJ*
O9	GCTTTGGGAATTCATTTAAACGAGATTGGGTGGA	*cylK*
O10	GCGGCAGCACCCGGGCGTGCCCTCCAATGTAGACTGCCTATT	*cylK*
O11	GGCACGCCCGGGTGCTGCCGCGATGGCTATATTTATGGTTATGCT	*cylK*
O12	AGTAAGGATCCCGTCTCTTTAATGCGG	*cylK*
O13	GTTTAGAATTCTTAGGCTTACTAACTTAGC	*Pcyl*
O14	CATTATTATGTTAAAATAGTAAC	*Pcyl*
O15	GTTACTATTTTAACATAATAATGTGTTTGAAGTAGATGTTTAGA	*Pcyl*
O16	TGTTAGGATCCACGACACTGCCATCAGCAC	*Pcyl*
O17	GTAAAGGGATCCGCTGTCTTGAAAGAGGCTATG	*cylK*
O18	AAGTATCTGCAGACTTAGCACTATTCGCATCA	*cylK*

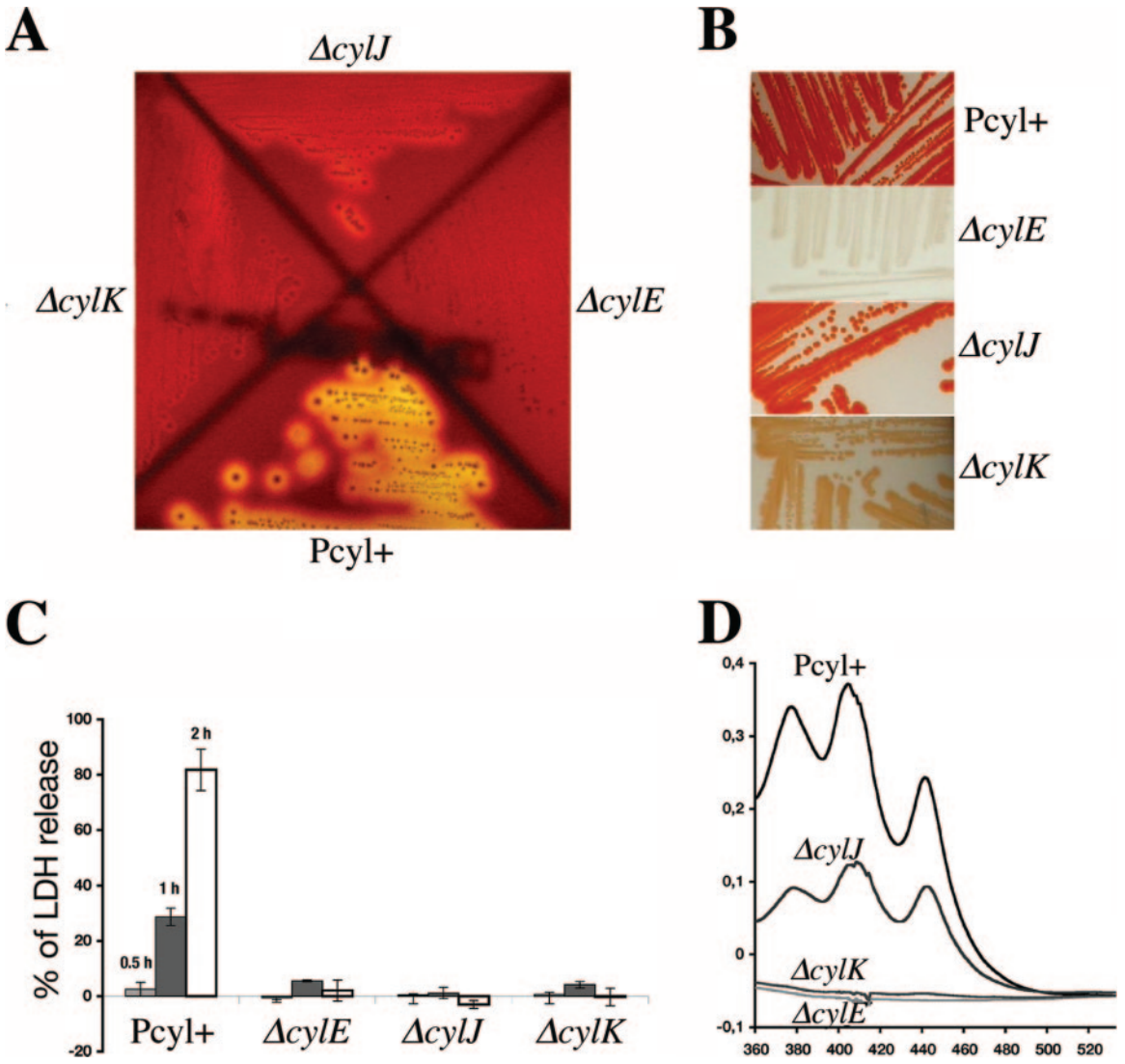

FIG. 2. Hemolytic/cytolytic activities and pigment formation in GBS strains. The in-frame deletion mutants NEM2456 (Δ*cylE*), NEM2457 (Δ*cylJ*), and NEM2458 (Δ*cylK*) derived from the hyperhemolytic and hyperpigmented strain CCH206 (Pcyl+). (A) Hemolytic phenotype of the mutants on horse blood agar plates (BioMérieux). (B) Pigment formation on Granada medium plates. (C) The cytolytic activity of GBS on A549 human alveolar epithelial cells (multiplicity of infection, 100 bacteria per cell) was assessed using the cytotoxicity detection kit (LDH) (Roche) at different time points (30 min, 1 h, and 2 h). The values were standardized such that the positive control phosphate-buffered saline–Triton 0.1% gave 100% of LDH release. (D) Absorbance profile of pigment extracts from the parental strain Pcyl+ and its Δ*cylE*, Δ*cylJ*, and Δ*cylK* isogenic mutants. The results shown are representative of three independent experiments.

GBS hemolysin and pigment biosynthesis and suggest a complex enzymatic pathway involving CylE as an essential component and CylJ and CylK as cofactors required for synthesis of a fully potent hemolysin. Polyene and fatty acid biosynthesis share common pathways (4). Of note, about half of the genes of the *cyl* operon code for enzymes involved in the biosynthesis of fatty acids (5), which can explain the close relationship found between hemolysin and pigment production in GBS.

CylE has been shown to be involved in GBS virulence (8), but the roles of CylJ and CylK have not been studied. Bacterial virulence levels were compared using a neonatal rat sepsis model (10), where the parental strain Pcyl+ and the isogenic Δ*cylE*, Δ*cylJ*, and Δ*cylK* mutant derivatives were injected intraperitoneally (i.p.) (Fig. 3). Randomized groups of 12 rat pups were inoculated i.p. with 100 μl of bacterial suspensions containing 5×10^6 GBS cells in 0.9% NaCl. The survival of the pups was monitored for 7 days, and the statistical significance of differences between groups observed was evaluated using the Mann-Whitney U test. A P value of <0.05 was considered statistically significant. The virulence of all mutants was significantly more attenuated than was that of the parental Pcyl+ strain, with the Δ*cylE* mutant being the less-virulent mutant (Fig. 3). Interestingly, the Δ*cylJ* and Δ*cylK* mutants displayed similar levels of virulence, which were intermediate to those of Pcyl+ and the Δ*cylE* mutant (Fig. 3). At day 7 postinfection, the percentages of mortality of the rat pups injected with Pcyl+ and the Δ*cylK*, Δ*cylJ*, and Δ*cylE* mutants were 90%, 75% ($P < 0.0213$), 70% ($P < 0.0213$), and 30% ($P < 0.0178$),

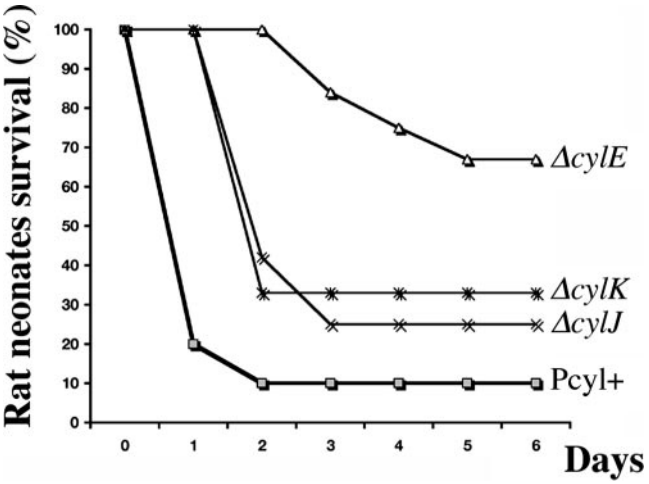

FIG. 3. Mortality curves in neonatal rats infected with the GBS parental strain Pcyl+ and its Δ*cylE*, Δ*cylJ*, and Δ*cylK* isogenic mutants. Two-day-old Sprague-Dawley rat pups (12 per strain) were inoculated i.p. with 5×10^6 bacteria.

respectively. Thus, these results seem to show a positive correlation between the virulence of these strains and their hemolytic activity.

In summary, we have studied the role of two uncharacterized genes, *cylJ* and *cylK*, belonging to the *cyl* operon. *cylJ*, the penultimate gene of the *cyl* operon, encodes a putative glycosyltransferase of 403 amino acids related to UDP-glucuronosyltransferase. *cylK*, the last gene of the *cyl* operon, encodes a 191-amino-acid protein and does not exhibit any similarity with other genes in the current databases. We showed that both *cylJ* and *cylK* are involved in, but not essential for, hemolytic/cytolytic activity and pigment production, two linked phenotypes in GBS, and that the level of hemolytic activity correlates with the virulence in a neonatal rat sepsis model. Elucidation of the biochemical nature of GBS hemolysin represents the next important challenge, and the CCH206 hyperhemolytic strain may be useful for that purpose.

We thank Josh Slater for the gift of the pCAM-45 SD1 plasmid and all the students of the Cours de Microbiologie Générale de l'Institut Pasteur 2005–2006 for the screening of the Himar1 transposon bank.

This work was supported by research funds from the Institut Pasteur (GPH no. 9) and the Centre National de la Recherche Scientifique (CNRS).

Editor: A. Camilli

REFERENCES

1. **Biswas, I., A. Gruss, S. D. Ehrlich, and E. Maguin.** 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J. Bacteriol. **175:**3628–3635.
2. **Dramsi, S., E. Caliot, I. Bonne, S. Guadagnini, M. C. Prevost, M. Kojadinovic, L. Lalioui, C. Poyart, and P. Trieu-Cuot.** 2006. Assembly and role of pili in group B streptococci. Mol. Microbiol. **60:**1401–1413.
3. **Glaser, P., C. Rusniok, C. Buchrieser, F. Chevalier, L. Frangeul, T. Msadek, M. Zouine, E. Couve, L. Lalioui, C. Poyart, P. Trieu-Cuot, and F. Kunst.** 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. Mol. Microbiol. **45:**1499–1513.
4. **Goel, A. K., L. Rajagopal, N. Nagesh, and R. V. Sonti.** 2002. Genetic locus encoding functions involved in biosynthesis and outer membrane localization of xanthomonadin in *Xanthomonas oryzae* pv. oryzae. J. Bacteriol. **184:**3539–3548.
5. **Gottschalk, B., G. Bröker, M. Kuhn, S. Aymanns, U. Gleich-Theurer, and B. Spellerberg.** 2006. Transport of multidrug resistance substrates by the *Streptococcus agalactiae* hemolysin transporter. J. Bacteriol. **188:**5984–5992.
6. **Liu, G. Y., K. S. Doran, T. Lawrence, N. Turkson, M. Puliti, L. Tissi, and V. Nizet.** 2004. Sword and shield: linked group B streptococcal beta-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. Proc. Natl. Acad. Sci. USA **101:**14491–14496.
7. **May, J. P., C. A. Walker, D. J. Maskell, and J. D. Slater.** 2004. Development of an in vivo *Himar1* transposon mutagenesis system for use in *Streptococcus equi* subsp. *equi*. FEMS Microbiol. Lett. **238:**401–409.
8. **Nizet, V.** 2002. Streptococcal beta-hemolysins: genetics and role in disease pathogenesis. Trends Microbiol. **10:**575–580.
9. **Nizet, V., R. L. Gibson, E. Y. Chi, P. E. Framson, M. Hulse, and C. E. Rubens.** 1996. Group B streptococcal beta-hemolysin expression is associated with injury of lung epithelial cells. Infect. Immun. **64:**3818–3826.
10. **Poyart, C., E. Pellegrini, M. Marceau, M. Baptista, F. Jaubert, M. C. Lamy, and P. Trieu-Cuot.** 2003. Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. Mol. Microbiol. **49:**1615–1625.
11. **Poyart, C., and P. Trieu-Cuot.** 1997. A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to beta-galactosidase in gram-positive bacteria. FEMS Microbiol. Lett. **156:**193–198.
12. **Pritzlaff, C. A., J. C. Chang, S. P. Kuo, G. S. Tamura, C. E. Rubens, and V. Nizet.** 2001. Genetic basis for the beta-haemolytic/cytolytic activity of group B streptococcus. Mol. Microbiol. **39:**236–247.
13. **Rosa-Fraile, M., J. Rodriguez-Granger, M. Cueto-Lopez, A. Sampedro, E. B. Gaye, J. M. Haro, and A. Andreu.** 1999. Use of Granada medium to detect group B streptococcal colonization in pregnant women. J. Clin. Microbiol. **37:**2674–2677.
14. **Rosa-Fraile, M., J. Rodríguez-Granger, A. Haidour-Benamin, J. M. Cuerva, and A. Sampedro.** 2006. Granadaene: proposed structure of the group B streptococcus polyenic pigment. Appl. Environ. Microbiol. **72:**6367–6370.
15. **Spellerberg, B., S. Martin, C. Brandt, and R. Lutticken.** 2000. The cyl genes of *Streptococcus agalactiae* are involved in the production of pigment. FEMS Microbiol. Lett. **188:**125–128.
16. **Spellerberg, B., B. Pohl, G. Haase, S. Martin, J. Weber-Heynemann, and R. Lütticken.** 1999. Identification of genetic determinants for the hemolytic activity of *Streptococcus agalactiae* by IS*S1* transposition. J. Bacteriol. **181:**3212–3219.
17. **Tapsall, J. W.** 1986. Pigment production by Lancefield-group-B streptococci (*Streptococcus agalactiae*). J. Med. Microbiol. **21:**75–81.